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Award Number: DAMD17-98-1-8035

TITLE: Potential Role of a Novel Nuclear Matrix Protein (nmt55) as a Tumor Marker in Human Breast Cancer Invasion and Metastases

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REPORT DATE: July 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2000	Annual Summary (1 Jul 99 - 30 Jun 00)

4. TITLE AND SUBTITLE
Potential Role of a Novel Nuclear Matrix Protein (nmt55) as a Tumor Marker in Human Breast Cancer Invasion and Metastases

5. FUNDING NUMBERS
DAMD17-98-1-8035

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

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13. ABSTRACT ***(Maximum 200 Words)***

The goal of this proposal is to test the hypothesis that loss of expression of a novel protein contributes to tumor growth, invasion and metastases, and its expression suppresses these biological events. We have identified, isolated and partially characterized a 55 kDa nuclear matrix protein from human breast tumor cells (hence forth referred to as nmt55). This novel protein is expressed in some estrogen receptor positive (ER+) tumors but was completely absent in ER- tumors. Loss of expression of this novel protein correlated strongly with tumor size ($p<0.03$) and loss of ER and PR ($p<0.001$). As the tumor size increased, the expression of nmt55 was not detected at the protein level. Because increased tumor size is associated with metastases, we postulate that loss of nmt55 expression is associated with molecular and cellular changes linked to cellular differentiation leading to loss of ER expression, and development of hormone-independent tumor growth, invasion and metastases. We have cloned the cDNA for nmt55 and generated site-directed polyclonal antibodies. We are currently investigating the function of nmt55 using biochemical and molecular biology approaches. The information derived from these studies will help determine the potential role of this novel nuclear matrix protein (nmt55) as a marker of tumor progression and metastases. These studies may provide critical information needed for early detection of potentially metastatic tumors, and improve diagnosis, prognosis and in developing strategies for therapeutic management and care of breast cancer patients.

14. SUBJECT TERMS
Breast Cancer
nmt55 Protein, Spicing Factor, Tumor Growth, Prognostic Marker

15. NUMBER OF PAGES
12

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Matthew Pavao 7-20-00

PI - Signature Date

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Biochemical and Molecular Characterization of a Novel Nuclear Matrix Protein in Human Breast Cancer: Relationship to Tumor Hormonal Status

Introduction:

The development of breast cancer is thought to be a multi-stage process (1). The progression of this disease is associated with cellular and molecular changes. Thus, initiation and progression may be related to loss of chromosomal material and ultimately specific gene function(s). Some of these cellular and molecular changes may be accompanied with tumor cell acquisition of metastatic potential. There is an urgent need for identification of node-negative patients whose tumors have metastatic potential. Several tumor markers have been used in assessing tumor changes linked to poor prognosis. These include loss of estrogen receptor (ER) and progesterone receptor (PR) expression (2), high blood vessel count (angiogenesis) (3), amplification of *erbB2/HER2/neu* gene (4) and decreased activity of *nm23* gene (5). None of these markers alone, however, predict with complete reliability which node-negative patients will likely relapse. We have found that primary human breast tumors express a 55 kDa nuclear protein, which is absent in estrogen receptor negative (ER-) tumors (6). This observation suggested that this protein may be related to tumor hormonal status and may represent a useful tumor marker. We have carried out preliminary studies to characterize this nuclear protein (referred to as *nmt55*) from human breast tumors and the MCF-7 cell line, using site-directed monoclonal antibodies and polyclonal antibodies. In this research, we have undertaken biochemical and molecular biology approaches to investigate the function(s) of this protein and its potential role in regulation of human breast cancer cell growth.

Results:

A. Detection of nmt55 Variants in Human Breast Tumors Using Western Blot and Immunohistochemical Analyses.

To further our investigation of nmt55 using molecular and biochemical approaches, our laboratory generated two epitope specific anti-peptide polyclonal antibodies (pAbs) to different domains of nmt55 to investigate the role of these domains in nmt55 function. One polyclonal antibody (pAb), termed NMT4, was raised against a unique peptide in the amino terminus of nmt55 and the second pAb, NMT5, was raised against a unique peptide in the mid-region of the protein. These antibodies, along with the monoclonal antibody (mAb) NMT1 (raised against the far carboxyl terminus of nmt55), were then used to screen various human breast tumors using Western blot or immunohistochemical Analyses.

When a series of tumors were screened via Western blot, the pAbs (NMT4 and NMT5) confirmed the original data observed with mAb NMT1 (6) indicating that ER- tumors displayed decreased nmt55 protein expression as compared to ER+ tumors. Interestingly, a subset of ER+ tumors expressed nmt55 protein that was not detected by pAb NMT4 but was detected by NMT5 and NMT1 antibodies using Western blot analyses.

To determine if this observation was the result of molecular changes within the tumor that altered nmt55 protein or rather an artifact of experimental preparation of breast tissue, we used the same set of antibodies to screen paraffin embedded human breast tumors by immunohistochemistry. As was observed with Western blot analysis, a subset of tumors analyzed by immunohistochemisty expressed nmt55 protein which was detectable only with pAb NMT5 and not with pAb NMT4. Since immunohistochemistry is a powerful tool used to investigate many proteins involved in breast cancer progression and is representative of the *in vivo* tumor environment, we suggest that nmt55 is indeed expressed as a variant, with alterations in its amino terminal domain, in a subset of human breast tumors. This alteration in the amino terminus of nmt55 may have deleterious effects with respect to nmt55 function and thus may play a role in breast cancer progression.

B. Intermolecular Association of nmt55 with the Polypyrimidine Tract Associated Splicing Factor (PSF).

The predicted nmt55 amino acid sequence suggested that nmt55 contains a bipartite RNA binding domain and posses strong homology to RNA binding proteins (6-9). This indicated that nmt55 may interact with other potential RNA binding proteins and may be involved in RNA processing. Data reported in year 1 of our study indicated that nmt55 could bind RNA via solubility experiments and that nmt55 interacted with a 100 kDa protein using immunoprecipitation experiments. Using specific antibodies we determined that this 100 kDa protein represented the well characterized polypyrimidine tract binding protein associated splicing factor (PSF) (7). These observations indicate that nmt55 may play an important role in the regulation of RNA processing and cellular function.

Since evidence suggested that nmt55 is also expressed in a variant form, we wanted to investigate the domain specific interaction of nmt55 with PSF. To implement in this study, we generated three GST-nmt55 fusion protein constructs. The first construct (GST-FL) encompassed the full-length nmt55 protein (residues 1-471) fused with GST protein, the second construct (GST-NT) fused the amino terminal portion of nmt55 protein (residues 1-227) with

GST protein and the final construct (GST-CT) fused the carboxyl terminal portion of nmt55 protein (residues 227-471) with GST protein. These constructs along with GST protein alone were incubated with MCF-7 cell nuclear extracts and subjected to GST pull-down assays and Western blot analysis. Using a specific PSF mAb, we showed that PSF interacts with both the amino terminus and carboxyl terminus of nmt55.

To determine if the nmt55/PSF interaction was specific for these two proteins alone, indicating multiple protein interactions, or rather that these proteins exist in a complex with other, as yet unidentified, proteins, we incubated our GST-nmt55 constructs with purified PSF protein and observed similar results. These results did not eliminate the possibility that nmt55 and PSF are part of a multi-protein complex but suggested that, regardless of other associated proteins, nmt55 and PSF have multiple molecular contacts. Thus, disruption of these contacts or alteration in domains responsible for these interactions may result in the loss of specific protein function.

C. Association of nmt55 with Several Essential pre-mRNA Splicing Factors.

The splicing and/or alternative splicing of pre-mRNAs is a critical regulatory step and, as a result, high efficiency of this step is essential in mammalian biology (11,12). It has been reported that as many as fifty different proteins may play critical roles in the splicing of pre-mRNAs (13). Since PSF has been characterized as an essential splicing factor (7) through binding to the polypyrimidine tract in pre-mRNAs, we investigated proteins that interact with the other essential pre-mRNA domains (the 5' splice site, branch A point and 3' splice site) for their ability to associate with nmt55. Reports indicate that several factors have been characterized and their functions partially elucidated (14). The SR (serine and argine rich) family of proteins has been shown to have a critical role in pre-mRNA splicing (15, 16). These proteins are involved in several RNA binding interactions involving all three critical pre-mRNA domains (17). Inhibition of these proteins has been shown to prevent *in vitro* pre-mRNA splicing (14-17). U1A 70K protein has been shown to interact with the U1snRNP and aid in its association with the 5' splice site on the pre-mRNA (18). $U2AF^{65}$ and $U2AF^{35}$ proteins are critical for the recruitment of the U2 snRNP to the branch A site and 3' splice site on the pre-mRNA, respectively (19, 20).

We investigated the ability of nmt55 to interact with members of the SR, U1 and U2 family using a mAb (16H3) raised against a series of serine and argine residues (21). Utilizing immunoprecipitation and Western blot analyses with mAb 16H3, we demonstrated that nmt55 interacts with a series of proteins in a large multi-protein complex. Several members of the SR family interact with nmt55 including SRp75, SRp55, SRp40 and SRp35. nmt55 also associates with U1 70K, $U2AF^{65}$, $U2AF^{35}$ and Topoisomerase I. Topoisomerase I has been shown to phosphorylate members of the SR protein family and may be important for RNA processing (22). The association of nmt55 with several well characterized and essential splicing factors strongly suggests a functional role for nmt55 in pre-mRNA splicing.

Summary and significance of the studies

In previous studies, we have demonstrated that nmt55 interacts with the RNA processing protein PSF and interacts with RNA, *in situ*. We have also shown that nmt55 protein expression is decreased in ER- human breast tumors. As a result of these observations, we developed specific antibodies to various domains of nmt55 and investigated the role of these domains in human breast tumors. We have identified nmt55 variants with alterations in their amino terminus in a subset of ER+ human breast tumors using Western blot and immunohistochemical analyses.

We have generated several GST-nmt55 fusion proteins to investigate the specific nmt55 domains responsible for PSF protein binding. We determined that nmt55 has multiple molecular contacts in its amino and carboxyl terminal domains with PSF protein. Along with PSF, we have elucidated the association of nmt55 with several essential splicing factors (U1 70K, U2AF^{65}, U2AF^{35}, SRp75, SRp55, SRp40, SRp35 and Topoisomerase I). These observations suggest that nmt55 may play an important role in RNA metabolism and/or processing.

The association of nmt55 expression with tumor hormonal status (ER and PR) in human breast tumors and its putative function as an RNA binding splicing suggests a key role in cellular growth and function. The data obtained suggests that nmt55 binds to RNA and many RNA splicing proteins and may have an important role in regulation of RNA metabolism. This may be critical in tumor cell growth and tumor progression.

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Appendix

Key Research Accomplishments

- We have detected nmt55 protein variants in a subset of human breast tumors. These nmt55 protein variants have alterations in their amino terminal region as determined by Western blot and immunohistochemical analyses using an epitope specific anti-peptide polyclonal antibody raised against the amino terminus of nmt55.

- We have determined the nmt55 domains responsible for protein interaction with the Polypyrimidine Tract Binding Protein Associated Splicing Factor (PSF). It was determined using GST-nmt55 fusion proteins and GST Pull Down Assays that nmt55 has multiple molecular contacts with PSF.

- We have identified a series of essential splicing factors (PSF, U1 70K, U2AF65, U2AF35, SRp75, SRp55, SRp40, SRp35 and Topoisomerase I) that associate with nmt55 in a multi-protein complex in human breast tumors and cell lines. These associations were determined by co-immunoprecipitation experiments coupled with Western blot analysis utilizing specific monoclonal and polyclonal antibodies.

Reportable Outcomes

Published Abstracts:

Pavao M, Moreland RB and Traish AM. "Potential Role of a Novel Nuclear Protein (nmt55) in Human Breast Cancer." Abstract, The FASEB Journal, 2000.

Poster Presentations:

Pavao M, Moreland RB and Traish AM. "Characterization of a Novel Nuclear Protein (nmt55) in Human Breast Cancer." Era of Hope: Department of Defense Breast Cancer Research Program. June 2000 (Atlanta, GA).

Pavao M, Moreland RB and Traish AM. "Potential Role of a Novel Nuclear Protein (nmt55) in Human Breast Cancer." American Society for Biochemistry and Molecular Biology. June 2000 (Boston, MA).

Pavao M, Moreland RB and Traish AM. "Identification and Characterization of a Novel Nuclear Protein (nmt55) in Human Breast Cancer." Henry I Russek Student Achievement Day. May 2000 (Boston, MA).

Pavao M, Moreland RB and Traish AM. "Characterization of nmt55 as a Potential Tumor Marker in Human Breast Cancer." Graduate Student Science Research Day. April 2000 (Boston, MA).

Pavao M, Moreland RB and Traish AM. "Potential Role of nmt55, a Novel Nuclear Protein, as a Tumor Marker in Human Breast Cancer Progression and Metastasis." Gordon Research Conference on Hormonal Carcinogenesis August 1999 (Tilton, NH)

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